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|THE INTERACTION OF 5'-FLUOROSULFONYL BENZOYL ADENOSINE
WITH IRON PROTEIN OF *AZOTOBACTER VINELANDII* NITROGENASE|

by

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INTRODUCTION

Nitrogenase, the enzyme system responsible for biological nitrogen fixation is found only among a minority of the procaryotic organisms. But the process it catalyzes is neverthless of great importance for life on earth; without it, the available supplies of combined nitrogen would soon be depleted by denitrification. Industrial fertilizers are energy costly, and although this is also true for biological nitrogen fixation, the organisms involved use solar energy more directly without intervening steps of technology.

Major research efforts on the biochemistry of nitrogen fixation are justified by curiosity about how enzyme proteins can carry out the difficult (for the chemists) process of reducing dinitrogen to ammonia under such mild conditions. The research is also justified by the need to define the biochemical requirements for nitrogen fixation before work in genetics, ecology and agronomy can be realistically expected to increase the number of useful nitrogen fixing system.

1. Nitrogenase and Its Structure

The nitrogenase complex consists of two proteins; Fe protein and MoFe protein. Although the two proteins can be completely

separated, and they can be obtained free of other proteins by standard techniques, neither of them is catalytically active in the absence of the other; these proteins must interact and form a complex during the catalysis. Both proteins are rapidly inactivated by oxygen (the Fe protein to the greater extent, half life in air about 30 sec), therefore they must be handled under strictly anaerobic conditions. Anaerobic techniques limit the number of experiments performed per nitrogenase enzymologist, and it causes another problem, that the presence of unknown portion of oxygen inactivated proteins introduces ambiguity in the data and their interpretations.

The Fe protein is a dimer, composed of two identical subunits. It has a molecular weight of about 60,000, containing four acid-labile sulfurs and four atoms which form one [4Fe 4S] cluster per Fe protein of two subunits (1). The [4Fe 4S] cluster bridges two identical subunits, being anchored through two cysteines on each subunit. Recently two particular cysteines (#97, #132) were identified as likely ligands to the [4Fe 4S] center (4). The amino acid sequence of Fe protein from eight different species has been determined (5-12). The amino acid sequences are highly conserved (13), although the organisms represent five genera (*Azotobacter*, *Clostridium*, *Anabaena*, *Klebsiella*, and *Rhizobium*) from diverse ecological niches and time of evolutionary divergence; the *C*. *pasteurianum* amino acid sequence is most different from all others, even it shares 60% residues in common with others. Crystal structure determination is in progress (14).

The MoFe protein is an $\alpha_1\beta_1$ tetramer with a molecular weight of 220,000 (1). It contains two atoms of molybdenum, and although the precise number of Fe atom is not known yet; the best values range from 24-32 (2). Acid labile sulfurs are present in about equal number as Fe. Isolation of FeMo cofactor (3), probably having a ratio of Fe to S to Mo of 8:6:1 and showing a similar electron paramagnetic resonance (epr) spectrum to that of intact MoFe protein, might be helpful for understanding the role of molybdenum at the active site and N_2 activating site in the MoFe protein.

2. Mechanism of Nitrogenase Catalysis

The conversion of dinitrogen to ammonia by nitrogenase complex requires both component proteins, a source of reducing equivalent, MgATP, protons, and anaerobic conditions. The stoichiometry of the reacion catalyzed by nitrogenase complex is

$$N_2 + 6e- + 12 \text{ MgATP} + 8 \text{ H}^+ \Rightarrow 2 \text{ NH}_4^+ + 12 \text{ MgADP} + 12 \text{Pi}$$

In most N fixing microorganisms, the sourse of electron is reduced ferridoxin. ATP and divalent metal ion, usually Mg, are absolute requirements for any electron transfer reactions by nitrogenase while MgADP, the product of MgATP utilization by enzyme, inhibits nitrogenase activity. Although there is not complete agreement, it is usually assumed that 12 moles of ATP are required for 1 mole of nitrogen reduced to 2 moles of ammonia (1). Relatively few details concerning regulation of the activity of nitrogenase are known.

Direct control of nitrogenase activity may be mediated through the ATP : ADP ratio in the vicinity of the enzyme thereby regulating the rate at which the enzyme reduces substrate (2).

(1) Turnover

The overall steps in nitrogenase catalysis have been reviewed (1). There are no known species different with respect to the overall steps, although the individual rate constant may vary. Mechanistic information has only come from studies with the artificial electron donor dithionite ($S_2O_4^{-2}$) where $SO_2 \cdot$ is the actual one electron donor. The overall steps are concisely summarized as follow. Fe is an abbreviation of Fe protein and MoFe is an abbreviation of MoFe protein.

(a) reduction of Fe protein by electron donor

$Fe_{ox}(MgADP)_2 + 2MgATP + SO_2 \cdot \Rightarrow Fe_{red}(MgATP)_2 + 2MgADP + SO_2$

(b) complex formation

$[Fe_{red}(MgATP)_2]_x + MoFe_{ox} \Rightarrow [Fe_{red}(MgATP)_2]_x \, MoFe_{ox}$

where x usually equal to 2 but can be 1 (15)

(c) electron transfer coupled to MgATP hydrolysis

$[Fe_{red}(MgATP)_2]_x \, MoFe_{ox} \implies [Fe_{ox}(MgADP + Pi)_2]_x \, MoFe_{red}$

There is a general agreement that this step is rapid and irreversible.

(d) complex dissociation

$[Fe_{ox}(MgADP + Pi)_2]_x MoFe_{red} \implies Fe_{ox}(MgADP)_2 + 2Pi + MoFe_{red}$
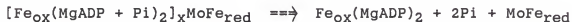
Free reduced MoFe protein might later transfer its electron to substrate, without the help of Fe protein.

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(2) Substrate Reduction

Besides dinitrogen, a number of other molecules and ions can be reduced through nitrogen catalysis, e.g., N_2O, N_3^-, C_2H_2, HCN, CH_3NC, H^+. Of these, the reduction of acetylene to ethylene is of great pratical importance for the measurement of nitrogenase *in vivo* and *in vitro* activity since this reduction can be easily estimated by gas chromatography. Reduction of protons to dihydrogen is also important since this substrate is always present with active nitrogenase systems.

3. Binding Of MgATP To The Nitrogenase Fe Protein

Electron transfer from Fe protein to MoFe protein is coupled to ATP hydrolysis. Relatively little is known about the role of MgATP in nitrogenase hydrolysis. Bui and Mortenson used gel filtration to show that ATP binds to Fe protein, but not to the MoFe protein (16). Tso and Burris extended equilibrium binding studies to quantitative experiments with a gel filtration technique (17). They found that ATP binds to two independent and equivalent sites on the Fe protein with a site-specific dissociation constant, Ks, 17 uM. ADP competes for binding for one site, but at the same time ADP increases the affinity of the other site for ATP. The dissociation constant for ADP is 5 uM.

Zumft et al (18) proposed that ATP induced a conformational change in Fe protein because the changes that ATP binding caused in the electron paramagnetic resonance (epr) spectrum were similar to

those spectral changes obtained when Fe protein was in the presence of 5.0 M urea.

Walker et at (19) reported that ATP-induced conformational change probably involved a reorientation of the iron sulfur center of Fe protein since the iron in reduced Fe protein was accessible to an iron chelator, α,α'- dipyridyl, in the presence of MgATP but not in its absence.

Hausinger and Howard (4) have shown that cys #85 residue is a likely nucleotide binding site. This residue is not a normally exposed, chemically reactive residue, but one which becomes exposed when iron-sulfer center is destroyed.

All these recent data support the idea that MgATP binding to the Fe protein dramatically influences the environment around the iron-sulfur center, which suggests close interaction between the MgATP binding site and iron sulfur center.

4. *In Vitro* Assay Of Nitrogenase Activity

Generally, for in vitro assays of nitrogenase activity, dithionite is used as an electron donor and the level of ATP is maintained high and nearly constant by using an ATP-regenerating system. The ATP-regenerating system consists of creatine phosphate, creatine kinase and set amount of ATP and Mg^{+2} and carries out the following reaction.

$$MgATP^{-2} + creatine \longleftrightarrow MgADP^- + creatine\ phosphate^{-2} + H^+$$
$$E:\ creatine\ kinase$$

It is assumed that the regenerating system responds in an ideal fashion so that the steady-state concentration of the essential substrate remains constant at the initial level while the concentration of inhibitor (e.g., MgADP) is negligible over the course of assay.

To meet the above purpose, the following conditions must be considered (20).

1) The effect of pH on the function of creatine kinase is complicated; at high pH the equilibrium is no longer overwhelmingly in the favor of ATP production and at low pH, the metal binding of nucleotides is reduced and dithionite is unstable. Therefore the pH between 7 and 8 must be maintained.

2) Mg as the acetate should be added in 1mM excess over [ATP] to ensure that all of ATP exists as the MgATP complex.

3) Cl^- or other commonly used anions inhibit creatine kinase activity.

5. Study Of Nitrogenase Using Nucleotide Analogs

Hausinger and Howard (4) identified two cysteines (#97, #132) as likely ligands to the iron sulfur center while another appeared to be involved in ATP binding (#85) using a chemical labeling method by alkylation with iodoacetic acid under various condition. Relatively few studies of covalent modification have been done with nitrogenase.

Robson (22) proposed that Fe protein has an ATP binding site

similar to that of many nucleotide binding enzymes. The specific amino acid residues within the nucleotide binding site of several enzymes were identified using affinity labeling by 5'-Fluorosulfonyl benzoyl adenosine (5'-FSBA) (23,24,25). The structure of 5'-FSBA (showm below) resembles that of ATP but it contains sulfonyl fluoride group which can act as an electrophilic agent in covalent reaction within several classes of amino acid including tyr, lys, his, ser, and cys. Thus it might reasonably be expected that 5'-FSBA would react with amino acid residues directly within adenine nucleotide binding sites in proteins (26). Therefore this affinity labeling reagent may be able to be used as a structural probe for nitrogenase. The studies in this paper are designed mainly to examine the interaction and kinetics of nitrogenase when it reacts with 5'-FSBA, before any attempt for a structural probe.
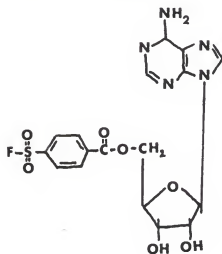
5'-Fluorosulfonyl benzoyl adenosine   Adenosine triphosphate
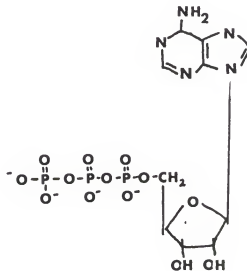
Source of chemicals

Adenosine triphosphate (ATP): Sigma Chemical Co.

Adenosine diphosphate (ADP): Sigma Chemical Co.

Creatine phosphate: Sigma Chemical Co.

Creatine kinase: Sigma Chemical Co.

5'-Fluorosulfonyl benzoyl adenosine (5'-FSBA): Sigma Chemical Co.

Phenylmethane sulfonyl fluoride (PMSF): Sigma Chemical Co.

Sodium dithionite (DTH): Fisher Scientific Co.

Calcium carbide (CaC_2): Fisher Scientific Co.

Trishydroxymethylaminomethane (Tris): Fisher Scientific Co.

37% (w/w) Formaldehyde (HCHO): Fisher Scientific Co.

2-(N-Morpholino)propane-sulfonic acid (MOPS): Sigma Chemical Co.

N, N - Dimethyl formamide (DMF): Fisher Scientific Co.

α,α'-Dipyridyl: J. T. Baker Chemical Co.

Mg acetate: Fisher Scientific Co.

1. Chemical Methods

(1) Acetylene Reduction Assay (21)

Materials

1. Standard ATP mix;

    315mg creatine phosphate

    48mg ATP

    6mg creatine kinase

    16.5ml 50mM Tris.Mops buffer, pH 7.4

    1.5ml 0.1M Mg acetate (MgAc)

2. Sodium dithionite solution: 100mM $Na_2S_2O_4$ (DTH)

    105mg sodium dithionite was dissolved in 6ml of 0.013N NaOH solution under anaerobic conditions prior to use. Before dissolving sodium dithionite with anaerobic NaOH, sodium dithionite must be capped with a rubber stopper and evacuated to replace air with argon gas under atmospheric pressure.

3. 37% (W/W) HCHO or 30% (W/V) trichloroacetic acid (TCA)

4. C_2H_2 gas;

    Acetylene produced from calcium carbide and water is used, which has adequate purity for acetylenereduction assay.

Methods

6.3 ml serum bottles were used for acetylene reduction assay. 1ml reation mixture contained 2.5 umole ATP, 30 umole

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creatine phosphate, 0.2 mg creatine kinase, 5 umole MgAc, and buffer; 0.6 ml of standard ATP mix satisfies all above concentrations. Bottles containing above constituents were evacuated and flushed 3 times with Ar gas. 0.5 ml acetylene gas and 20 umole dithionite solution was added just before the addition of enzyme fraction. Assays were run at $30^{\circ}C$ for an indicated time in a water-bath shaker. Acetylene reduction reactions were terminated by injecting 0.1ml of 30% TCA or 37% HCHO to the solution. Ethylene production was measured after 30 min with a Varian Aerograph 1200 gas chromatograph.

Sample peak squares can be converted to nmole, calibrating with an ethylene standard peak. The way to get the standard peak is described; ethylene and acetylene gas, each one mililiter, were injected into 125ml vaccine bottle capped with a rubber stopper. The amount of acetylene and ethylene in this bottle is calculated as follows.

$$1ml/24,000ml/mole \quad x \quad 1ml/125ml \quad = \quad 333 \times 10^{-9} \text{ mole/ml}$$

Therefore, if 300 ul of the bottle content is injected, it must give 100 nmole of both acetylene and ethylene.

$$333 \times 10^{-9} \text{mole/ml} \times 300 \text{ ul} \times 10^{-3} \text{ml/1} \quad = 99.9 \times 10^{-9} \text{ mole}$$
$$= 100 \text{ nmole}$$

To get a standard peak, 300 ul of the bottle contents was injected into Varian Aerograph 1200 and peaks were counted by their squares. To get sample peak, 500 ul gaseous phase was injected, peaks were counted, and converted to nmole per sample as shown next.

calibration factor x concentration (nmole) x dilution factor
= nmole/sample bottle

where

$$\text{calibration factor} = \frac{\text{square x attenuator x sensitivity of sample}}{\text{square x attenuator x sensitivity of std.}}$$

concentrations = 100 nmole

$$\text{dilution factor} = \frac{\text{total vial vol} - \text{liquid vol}}{\text{sample size}} = \frac{6.3 - 1.0 \text{ ml}}{0.5 \text{ ml}}$$

In the experiment performed for this paper, all the activity was shown as reduced C_2H_2 nmole/min/assay and each assay condition was as descrived above. The gas chromatograph detector was hydrogen flame ionization detector and the carrier gas was nitrogen. The column temperature was at 70°C, injector temperature at 38°C, and detector temperature at 130°C.

< Enzyme titration>

Acetylene reduction activity is dependent on the ratio of two components of nitrogenase as well as C_2H_2 concentration (27). It is necessary to titrate one component against the other until optimum specific activity based on both components is reached to get a suitable system for kinetic studies of nitrogenase.

Methods

Six reaction vials containing reagents except AV II were prepared by the following protocol anaerobically and incubated at 30° C for 6 minutes. As per time schedule, ten ul of component II was added and after 5 minutes the reaction was stopped and the activity was measured. The enzyme titration curve, the plot of measured activity vs the amount of added component I provides the information about the component ratio to be used.

run	ATP mix	100mM DTH	C_2H_2 gas	H_2O	buffer	comp I	comp II
						(unit: ul)	
1	600	200	500	100	90	0	10
2					85	5	
3					80	10	
4					75	15	
5					65	25	
6					75	25	0

α,α'- dipyridyl (structure shown below) is a reagent for the determination of iron; it chelates ferrous iron resulting in a colored product, a ferrous α,α'-dipyridyl complex which has a molar extinction 8400 at 520 nm. It has been proposed that the reason that these compounds form such stable complexes with Fe is that iron-chelator-complex exists in the form of five membered aromatic ring capable of assuming several resonance structures (28).

R = N,O

This color reaction was exploited to determine Fe of Fe protein in various conditions. To protect enzyme from being denatured by oxygen, absorbance was measured anaerobically by Beckman UV spectrometer.
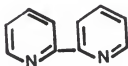
structure of α,α'-dipyridyl

2. Enzyme Studies

The enzyme used in this experiment was isolated from *Azotobacter vinelandii* which are free living bacteria, being able to fix nitrogen without the cooperation of other organisms. For the nitrogenase system of *Azotobacter vinelandii*, component I and II are commonly denoted by AV 1 and AV II, respectively. The enzymes used in this experiment were obtained from several different vessels which were isolated as different fractions, at different preparation date. Therefore for each different vessel of enzyme, enzyme titration was performed to get the suitable component ratio to be used.

(1) 5'-FSBA Effect On Each Separated Component

This experiment is designed to determine whether 5'-FSBA has a specific inhibition of either component I or II from *Azotobacter vinelandii* or whether both need to be present for the inhibition. Since Dr. Davis's earlier observation (unpublished) showed that 5'-FSBA inhibition was stronger in the presence of MgADP than 5'-FSBA alone, all the samples were reacted in the presence of MgADP.

Reagents:

    1 standard ATP mix

    2. 100 mM sodium dithionite solution (DTH)

    3. acetylene gas

    4. 37% (W/W) HCHO

    5. 50 mM Tris Mops buffer, pH 7.4

    * all above are prepared as described in chemical methods.

    6. N,N – Dimethyl formamide (DMF)

    7. 25 mM 5'-FSBA solution:

        2.45 mg of 5'-FSBA was dissolved in 0.2 ml of DMF solution anaerobically. Since 5'-FSBA was not stable in DMF solution for a long time, fresh solution was prepared prior to use.

    8. 100 mM Mg acetate in distilled water

    9. 50 mM ADP solution in distilled water

    10. AV I solution

    11. AV II solution

EXPERIMENT (1)-a, 5'-FSBA effect on AV I

(The enzyme used was AV I, 5/5/81, A #5 and AV II, 5/4/81, A #1)

Table 1. Enzyme Titration Data

```
------------------------------------------------------------
   AVI (ul)      AVII (ul)        activity (nmole/min)
------------------------------------------------------------
     0             10                    0.58

     5              |                    6.99

    10              |                   13.30

    15              |                   14.10

    25              ↓                   17.60

    25              0                    0.34
------------------------------------------------------------
```

     Maximum activity was about 2,000 nmole/min/ml AV II and the
ratio of components, the volume ratio present in assay, was
determined to have AV I to AV II (1 to 2) in order that enzyme
activity was limited by AV I, not by AV II.

Procedure:

     The reagents were prepared as shown in following protocol
anaerobically and incubated in water-bath shaker at 30°C for 6 min.

```
----------------------------------------------------------------
 run   buffer   0.1M MgAc  50mM ADP  100mM DTH  DMF  25mM FSBA
----------------------------------------------------------------
                                                 (unit: ul)
  1     125        5          5         50       40     --

  2     125        5          5         50       --     40
----------------------------------------------------------------
```

Time was measured by adding 65 ul of AV I. At 0, 10, 30 ,60,

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120min, 40 ul reaction mixture was taken out and added to enzyme assay vials which contained 300 ul ATP mix, 40 ul buffer, 100 ul DTH, 20 ul AV II and 500 ul C_2H_2, incubating in water bath. After 5 min of addition of reaction mixture (containing 9 ul of AV I) to the enzyme assay vial, reaction was stopped by adding 0.1 ml of 37% HCHO solution and 500 ul gas sample was injected into gas chromatograph.

EXPERIMENT (1)-b   5'-FSBA effect on AV II

(The enzyme used was AV I, 5/5/81, B #4 and AV II, 4/16/80)

Table 2 Enzyme Titration Data

AV I (ul)	AV II (ul)	activity (nmole/min)
0	10	0.18
5		6.40
10		9.60
15		13.90
25		13.20
25	0	0.00

Maximum activity was about 1,400 nmole/min/ml AV II and enzyme component volume ratio was determined to have excess of AV I over AV II (2.8 : 1) in order that enzyme activity was limited by AV II, not by AV I.

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Procedure:

Same as experiment 1-a except that AV II (65 ul) was added to the reaction bottle instead of AV I and AV I (25 ul) instead of AV II (20 ul) was added to enzyme assay bottle.

(2) 5'-FSBA Effect On Mixed Components

In experiment (1), 5'-FSBA showed specific inhibition on AV II. This experiment was designed to determine whether 5'-FSBA has an inhibition effect on AV II when it exists in a mixed form with AV I.

Reagents:

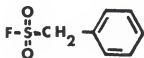same as experiment (1)-b except enzyme solution.

Instead of separated components, enzyme mixture containing AV I and AV II at a volume ratio of 2.5 to 1 was used.

Procedure:

All steps are the same as experiment (1)-b except that the enzyme mixture (65 ul) instead of AV II was added to reaction bottles and enzyme assay bottles contained the same amount of buffer instead of AV I.

(3) Phenylmethane Sulfonyl Fluoride (PMSF) Effect On AV II

This experiment was designed to know the specifity of 5'-FSBA inhibition; was 5'-FSBA inhibition on AV II due to being a structural analog of ATP or just due to a reactive sulfonyl fluoride group? For this purpose, PMSF (phenylmethane sulfonyl fluoride, structure was shown below) which has the same reactive sulfonyl fluoride group as 5'-FSBA and inhibits the serine protease by reacting with active amino acid residue (29) was reacted with AV II and its effect on enzyme activity was determined.

structure of phenylmethane sulfonyl fluoride (PMSF)

Reagents:

Same as experiment (1) except 5-FSBA solution and enzyme solution. Instead of 25 mM 5'-FSBA, 25 mM PMSF solution was prepare in DMF solution. The enzyme solution used was AV I 5/5/81, B #4 and AV II, 5/4/81, B #1.

Table 3. Enzyme titration data

AV I (ul)	AV II (ul)	Activity (nmole/min)
0	10	0.3
5		4.8
10		8.59
15		9.69
25	↓	12.20
25	0	0.02

Maximum activity was more than 1,300 nmole/min/ml AV II and the volume ratio of AV I to AV II (2 to 5) was used.

Procedure:

The reagents were prepared in reaction vials as shown in following protocol and placed in water-bath shaker at 30°C.

run	buffer	100mM DTH	0.1M MgAc	50mM ADP	DMF	25mM PMSF
						(unit: ui)
1	225	50	10	10	80	--
2	225	50	10	10	--	80

Time was measured by adding 125 ul of AV II. AT 0, 10, 30, 60, 120 minutes, 40 ul reaction mixture was taken out and added to enzyme assay vials which contain 300 ul ATP mix, 35 ul buffer, 100 ul DTH, 25 ul AV I and 500 ul C_2H_2 gas, incubating in water-bath shaker. After 5 minutes of addition of reaction mixture, reaction was stopped and activity was measured.

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(4) 5'-FSBA Inhibition With MgADP

This experiment was designed to determine 5'-FSBA inhibition with MgADP in standard assay medium. In the enzyme assay method of experiment (1) and (2), enzyme was incubated with 5'-FSBA and MgADP for a certain indicated time and small aliquots of reaction mixture were taken out and added to enzyme assay bottle to measure activity. In this experiment, to measure continuing turnover of catalysis upon 5'-FSBA inhibition, the reaction bottle and enzyme assay bottle were not separated. To incubate enzyme with 5'-FSBA in the presence of MgADP, standard ATP mix was modified as described in reagents. However, nitrogenase enzyme activity was not measurable without MgATP, so after different incubation time with MgADP, creatine kinase solution was added and activity was measured.

Reagents:

1. 2X ADP mix

creatine phosphate	620mg
ADP	72.8mg
Mg acetate	3ml
50mM Tris Mops buffer, pH 7.4	15ml

(300 ul of 2X ADP mix in 1 ml reagent volume satisfies 2.5 umole ADP, 30 umolecreatine phosphate and 5umole Mg acetate which need for enzyme assay.)

2. Creatine kinase solution (10mg/ml) in 50 mM Tris Mops buffer (20 ul of kinase solution gives 0.2 mg to 1 ml

reaction mixure which needs for enzyme assay.)   All the other
reagents used including enzyme solution were the   same as
experiment (1).

Procedure:

The following reagents were prepared in reaction vials
anaerobically and incubated in 30°C water-bath for 6 min.

run	2X ADP mix	buffer	100mM DTH	AV I	C_2H_2	H_2O	DMF	25mM FSBA
							(unit: ul)	
1	300	110	200	150	500	120	40	--
2	300	110	200	150	500	120	---	40

total liquid volume, 1ml

Each run is comprised of six reaction vials. As per time schedule,
60 ul of AV II was added into the reaction vials which were placed
in water-bath shaker during reaction. After each time interval (0,
10, 15, 30, 60, 90 min), 20 ul creatine kinase solution was added
to convert MgADP to MgATP and enzyme activity was measured for 5
minutes. 500 ul gaseous sample was taken out and injected to gas
chromatograph.

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(5) Time Course Of 5'-FSBA Inhibition

In experiment 4, 5'-FSBA showed a strong inhibition in 10 minutes. This experiment was designed to determine the initial inhibition by 5'-FSBA; the enzyme activity was monitored in every 1 min up to 5 min in two different ATP concentrations. Instead of standard ATP mix, each constituent was made separately in order to vary ATP concentration.

Reagents:

1. 200 mM creatine phosphate prepared in 50 mM Tris Mops buffer

2. 50 mM ATP solution prepared in 50 mM Tris Mops buffer

3. 100 mM Mg acetate solution prepared in distilled water

4. Creatine kinase solution (10 mg/ml) in 50 mM Tris Mops buffer

All the other reagents were same as experiment (4). The enzyme solution used was AV I, 5/5/81, A #4 and AV II, 5/4/81, B #1)

Table 4 Enzyme Titration Data

AV I (ul)	AV II (ul)	activity (nmole/min)
0	10	0.12
5		3.34
10		4.61
15		4.49
25		3.36
25	0	0.52

Maximum activity was about 500 nmole/min/ml AV II and the volume ratio of AV I to AV II (1 to 1) was used to have excess AV I over AV II.

Procedure:

The reagents were prepared in reaction vials by the following protocol under anaerobic conditions and placed in water-bath shaker at 30°C for 6 min.

	0.75 mM ATP 0.5 mM FSBA	1.5 mM ATP 0.5 mM FSBA
		(unit: ul)
200 mM creatine P	150	150
100 mM Mg acetate	50	50
H_2O	145	145
100 mM DTH	200	200
C_2H_2	500	500
50 mM ATP	15	30
buffer	300	285
AV I	50	50
DMF or 25 mM FSBA	20	20

Each ATP concentration is comprised of five reaction vials for 5' − FSBA treated and one reaction vial for DMF control. As per time schedule, 20 ul of creatine kinase solution was added and immediately after (10sec), 50 ul AV II was added into the reaction

vial which was in water-bath shaker. For the 5'-FSBA treated vials, after each time interval (1, 2, 3, 4, 5 min after adding AV II), reaction was stopped by adding 37% HCHO and activity was measured. For the DMF control vials, reaction was stopped after 5 min of addition of AV II.

(6) Initial Fast Inhibition As A Function Of [5'-FSBA] or [ATP]

In experiment (5), 5'-FSBA inhibition was occurring in two different phases; up to 1 min, the inhibition was occurring very fast and later it was rather slow. This experiment was designed to determine the fast inhibition as a function of ATP concentration or 5'-FSBA concentration. The fast inhibition seems to occur instantaneously, but in this experiment the reaction up to 2 min was assumed as a reaction containing the initial fast inhibition for practical reasons.

EXPERIMENT (6)-a. 5'-FSBA inhibition as a function of [ATP]

Reagents:

1. 50 mM ATP and 50 mM Mg acetate solution, pH 7.4.

Since pH could decrease in high [ATP] or [Mg acetate] and decreased pH could affect the equilibrium of creatine kinase, mixture of 50 mM ATP and Mg acetate was prepared adjusting pH to 7.4 by adding 1 M Tris solution.

All the other reagents used were same as experiment 5. The enzyme solution used was AV I, 5/5/80, A #4 and AV II,4/16/80.

Table 5. Enzyme Titration Data

AV I (ul)	AV II (ul)	activity (nmole/min)
0	10	0.1
5	⎥	7.9
10	⎥	14.46
15	⎥	17.48
25	↓	24.88
25	0	0.0

Maximum activity was more that 2,500 nmole/min/ml AV II and the volume ratio of AVI to AVII (2.5 to 1) was used.

Procedure:

The reagents were prepared by the following protocol under anaerobic conditions and incubated in water-bath shaker at $30^{\circ}C$ for 6 min.

[ATP] (mM)	0.25	1.0	1.5	2.5	5.0	7.5	10.0
					(unit: ul)		
creatine phosphate	150						
H_2O	145						
100 mM DTH	200			all	same		
C_2H_2 gas	500						
100 mM Mg acetate	10						
AV I	125						
50 mM ATP & MgAc	5	20	30	50	100	150	200
buffer	255	240	230	210	160	110	60
DMF or 25 mM FSBA	40			all	same		

To get a relative inhibition, each ATP concentration is comprised
of two reaction vials; one for DMF control and the other for 5'-
FSBA treated. 20 ul creatine kinase was added and immediatly after
50 ul AV II was added. After 2 min, reaction was stopped and
activity was measured.

EXPERIMENT (6)-b, Initial fast inhibition as a fuction of [5'-
FSBA]

Reagents:

   same as experiment (5), except enzyme solution

   Instead of separated components, enzyme mixture containing AVI
   and AV II at a ratio of 4 to 1 was used. The enzyme used was
   AV I: 5/5/81, A #1 and AV II: 5/4/81, A #1.

Table 6. Enzyme Titration Data

AV I (ul)	AV II (ul)	activity (nmole/min)
0	10	0.01
5		6.05
10		9.26
15		9.09
25		12.63
25	0	0.03

Maximum activity was more than 1,300 nmole/min/ml and the volume
ratio of AV I to AV II (4 to 1) was used to have excess AV I over

AV II.

Procedure:

 Five reaction vials for each ATP concentration were prepared as following protocol anaerobically. As per time schedule, 20 ul creatine kinase solution was added and immediately after (10sec) 50 ul enzyme mixture was added into reaction vials which were being in water-bath shaker. After 2 min of addition of enzyme solution, reaction was stopped and activity was measured by gas chromatograph.

[5'-FSBA](mM)	0.25 (0.5 or 1.0 mM) ATP				
	0	0.25	0.5	1.0	1.5
				(unit:ul)	
creatine P	150				
H_2O	180				
C_2H_2	500		all same		
100 mM DTH	200				
100 mM Mg acetate	50				
50 mM ATP	10	(20	or	• 40)	
buffer	280	(270	or	250)	
DMF	60	50	40	20	0
25 mM 5'-FSBA	0	10	20	40	60

(7) Rate Of Irreversible Inhibition

Experiment (6) showed that 5'-FSBA inhibition on AV II was ATP concentration dependent; high concentration of ATP prevented the inhibition by 5'-FSBA. This experiment was designed to get the rate of irreversible inhibition exploiting above information. First, AV II was reacted with 5'-FSBA at 0.5 mM of [ATP] and activity was monitored in every one min up to 5 min. Next, AV II was reacted with 5'-FSBA at 0.5 mM ATP and in every one minute instead of terminating reaction, high concentration of ATP stock solution was added to increase ATP concentration to 2.5 mM and reaction was terminated after 5 minutes of addition of ATP stock solution. The difference in activity between theses two reactions could tell the remaining activity at higher concentration of ATP and the rate of the remaining activity could tell the rate of irreversible inhibition that is the inhibition still left at high concentration of ATP.

Reagents:

   same as experiment (6)-a

   Also same enzymes were used.

Procedure:

         Ten reaction vials containing creatine phosphate (150 ul), H_2O (180 ul), 100 mM DTH (200 ul), C_2H_2 gas (500 ul), 50 mM ATP (10 ul), 100 mM Mg acetate (10 ul), buffer (300 ul),

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and 25 mM 5'-FSBA (25 ul) were prepared anaerobically. For five reaction vials, as per time schedule, 20 ul of creatine kinase was added and immediately after (10sec) 50 ul of enzyme was added. After each time interval (1, 2, 3, 4, 5min after addition of AV II), the reaction was stopped. For the remaining five reaction vials, the same procedures were followed except that at each time interval (1, 2, 3, 4, 5min) instead of terminating the reaction, 40 ul of 50 mM ATP stock solution was added to increase [ATP] to 2.5 mM and reaction was stopped after 5 minutes.

For a DMF control experiment, one reaction vial containing 40 ul of DMF instead of 5'-FSBA at 2.5 mM was prepared. 20 ul creatine kinase was added and immediately 50 ul enzyme mixture was added. 5 minutes later reaction was stopped and activity was measured.

(8) Fe Determination Of AV II In The Presence Of 5'-FSBA By α,α'-Dipyridyl

This experiment was designed to determine whether inhibition by 5'-FSBA on AV II is related with iron sulfur center of Fe protein; if bound 5'-FSBA reacts with iron-sulfur center and alters iron-sulfur center reactivity with chelator, α,α'-dipyridyl, then different amount of Fe could be detected by α,α'-dipyridyl in the presence of 5'-FSBA.

Reagents:

1. 50 mM Tris HCl buffer, pH 8.0 containing 0.1 M NaCl
2. 20 mM α,α' - dipyridyl in 50 mM Tris HCl buffer was prepared anaerobically.
3. 25 mM 5'-FSBA in DMF was prepared anaerobically.
4. N, N - Dimethyl formamide (DMF)
5. 0.1 M Mg acetate solutition in distilled water
6. 50 mM ADP solution in distilled water
7. 100 mM sodium dithionite solution (DTH)
8. AV II

Procedure:

run	100mM MgAc	50mM ADP	buffer	100mM DTH	AV II	20 mM α,α'-dipyridyl	DMF	25mM FSBA
							(unit: ul)	
1	10	10	230	10	500	200	40	--
2	10	10	230	10	500	200	--	40

In two cuvettes, the first three constituents in above protocol were prepared anaerobically, capped tightly using a serum stopper and evacuated, flushed with Ar gas three times. DTH and enzyme solution were added using a syringe anaerobically and absorbance at 520 nm was measured for blank. Next, α,α' -dipyridyl was added and absorbance was measured to preexisting inactivated AV II. DMF or 5'-FSBA solution was added and aborbance was continuously measured up to 90 minutes.

Enzyme Studies

(1) 5'-FSBA Effect On Each Separated Component

Fig (1) and (2) showed that 5'-FSBA had a specific inhibition effect on AV II but not on AV I. Since Dr. Davis's earlier observation (unpublished) showed that 5'FSBA inhibition effect was stronger with MgADP than 5'-FSBA alone, enzyme was reacted with 5'-FSBA in the presence of MgADP. Almost all of the nitrogenase activity was inhibited in 2 hours. 5'-FSBA showed an inhibition effect when a small aliquot of AV II reacted with 5'-FSBA was diluted into large volume in an assay bottle, indicating that 5'-FSBA inhibits AV II irreversibly.

N, N – Dimethyl formamide (DMF), the organic solvent used to dissolve 5'-FSBA showed a litte inhibition effect on nitrogenase activity, but not any considerable amount. One way to minimize the DMF effect is to increase 5'-FSBA stock solution concentration in order to reduce the amount of DMF solution to be used. 25 mM 5'-FSBA in DMF was likely to be the highest concentration due to appearance of precipitate in higher concentration. Although I could increase the 5'-FSBA concentration to 50 mM by dissolving precipitate in hot water-bath, sometimes 5'-FSBA did not show the inhibition effect at that concentration, indicating sulfonyl fluoride group might be hydrolyzed.

Figure (1)-a. 5'-FSBA effect on AV I

    Figure (1)-a shows 5'-FSBA effect on AV I; 3.4 mM of 5'-FSBA
was reacted with AV I in the presence of 1.0 mM ADP and   2.0 mM
Mg. After indicated reaction time of AV I with 5'-FSBA,   a 40
ul aliquot of reaction mixture (containing 9 ul of AV I) was
added to enzyme assay vials (containing 20 ul of AV II) and
assayed for 5 minutes.

          O : DMF control
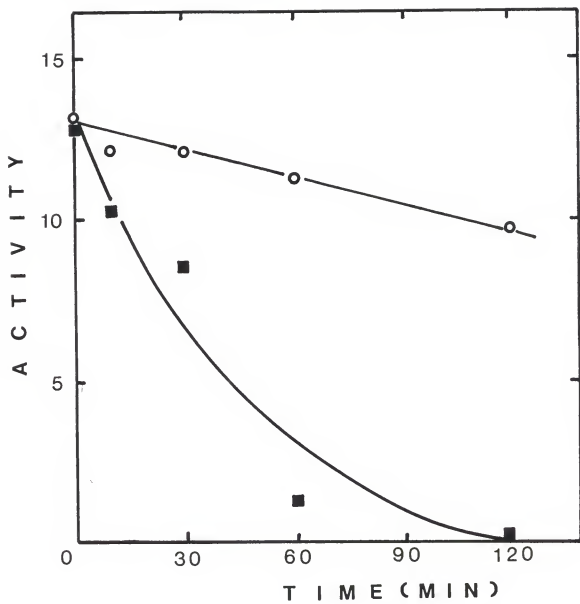   ■ : 5'-FSBA treated
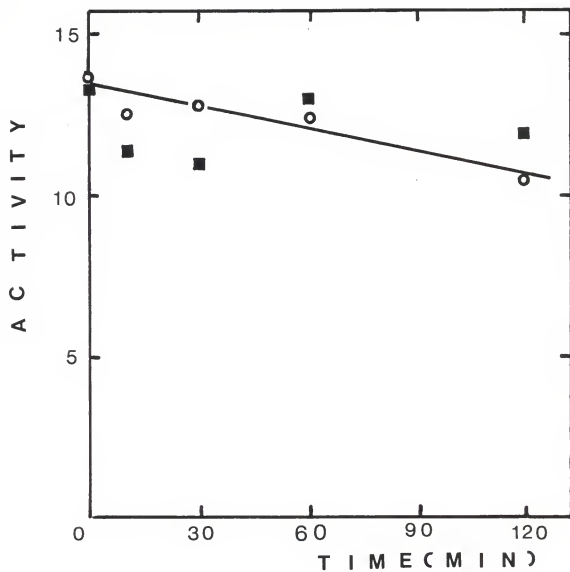
Figure (1)-b. 5'-FSBA effect on AV II

Figure (1)-b shows 5'-FSBA effect on AV II; 3.4 mM of 5'-FSBA was reacted with AV II in the presence of 1.0 mM ADP and 2.0 mM of Mg acetate. At the indicated time, a 40 ul aliquot of reaction mixture containg 9 ul of AVII was added to enzyme assay vials containing 25 ul of AV I and assayed for 5 minutes.

        O   :   DMF control

        ■   :   5'FSBA treated

(2) 5'-FSBA Effect On Mixed Components

     5'-FSBA inhibition effect on mixed components was tested for the purpose of practical use. Figure 2 showed that 5'-FSBA had same inhibition effect on AV II when AV II was present with AV I in a mixture. This result allowed me to be able to use the enzyme solution in a mixture of both components in order to cut off one adding step, not necessarily reacting AV II with 5'-FSBA and combining with AV I.

LEGEND to Figure (2)

Figure (2). 5'-FSBA effect on mixed components

      Figure (2) shows 5'-FSBA effect on enzyme mixture; 3.4 mM
5'-FSBA was reacted with 65 ul of enzyme mixture (containing AV I
to AV II at a volume ratio of 2.5 to 1) in the presence of 1.0 mM
ADP and 2.0 mM Mg acetate. After an indicated incubation time, a 40
ul aliquot containing 9 ul enzyme mixture was added to enzyme assay
vials and assayed for 5 minutes at 30°C. Activity was shown as
reduced C_2H_2 nmole/min/assay.

    O   : DMF control
    ■   : 5'-FSBA treated

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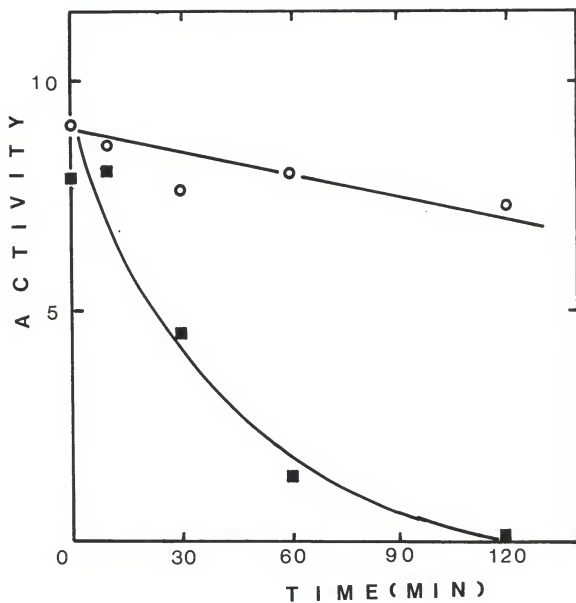
(3) PMSF Effect On AV II

Figure 3 shows the PMSF effect on AV II; although DMF control was not very constant, the enzyme activity of PMSF treated changed almost same rate as control, indicating that PMSF did not have any inhibition effect on AV II.

This result that PMSF which contains same reactive sulfonyl fluoride group as 5'-FSBA did not inhibit AV II while 5'-FSBA had an inhibition effect on AV II suggested that 5'-FSBA specifically inhibited enzyme because not only it had a reactive sulfonyl fluoride group but also it was a structural analogy to ATP.

LEGEND to Figure 3

Figure 3. PMSF effect on AV II

     Figure 3 shows PMSF effect on AV II; 1 mM of PMSF was reacted with AV II in the presence of 1.0 mM ATP and 2.0 mM of MgAc. After indicated reaction time of AV II with PMSF, a 40 ul aliquot of reaction mixture (containing 10 ul of AV II) was added to enzyme assay vial (containing 25 ul of AV I) and assayed for 5 minutes.

          O ; DMF control

          ■ ; PMSF treated
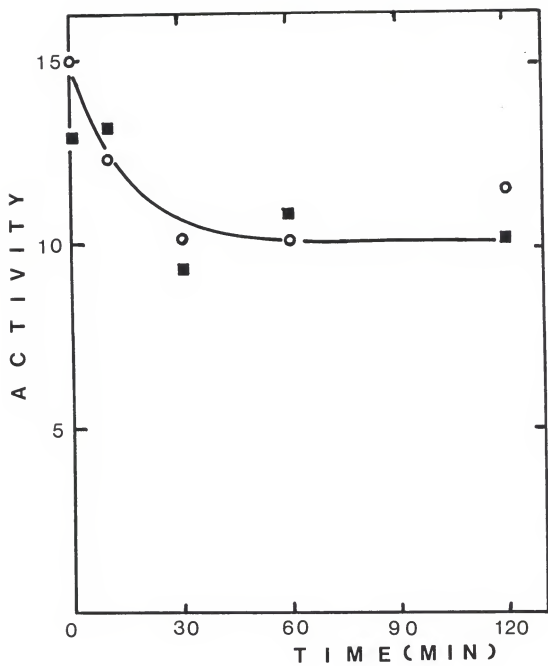
(4) 5'-FSBA Inhibition With MgADP

As shown in Figure (4), 5'-FSBA inhibition with MgADP in the standard assay medium was much faster than that of experiment 1 or 2; about 90 % of nitrogenase activity was inhibited in 10 minutes and in 30 minutes almost all of the activity was inhibited comparing the result of experiment 1 where 20 % activity was inhibited in 10 minutes and in 2 hours all of the activity was inhibited. Continuing enzyme turnover in the standard assay medium might facilitate the 5'-FSBA inhibition effect.

Figure 4. 5'-FSBA inhibition with MgADP

      Figure 4 shows the 5'-FSBA inhibition effect with MgADP in standard assay medium. 60 ul of AV II was incubated with 1 mM of 5'- FSBA in the presence of 2.5 mM ADP and 5.0 mM MgAc. To measure inhibition at standatd enzyme assay concentrations, 150 ul of AV I and the other reagents for assay were added in same vial except creatine kinase to prevent the conversion of MgADP to MgATP. After an indicated incubation time with MgADP, enzyme activity was measured for 5 minutes by adding 20 ul of creatine kinase stock solution (10mg/ml).

         O : DMF control

         ■ : 1 mM 5'-FSBA
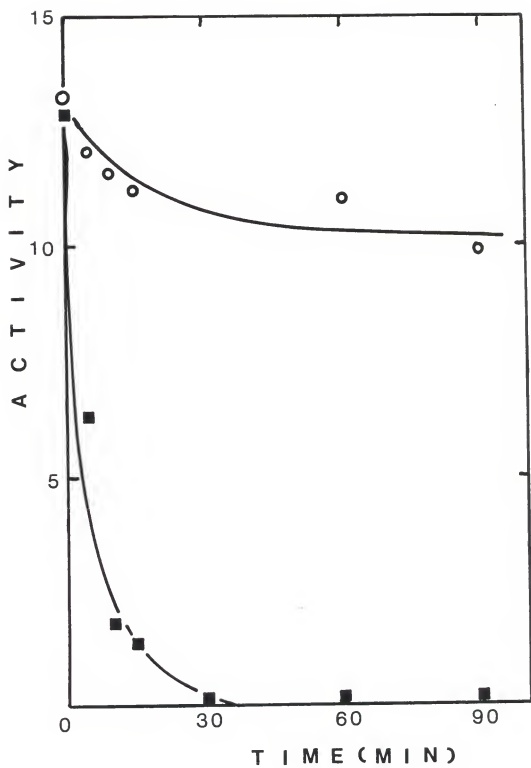
(5) Time Course Of 5'-FSBA Inhibition

Figure (5)-a showed the result of time course activity in two different ATP concentrations (0.75 mM and 1.5 mM) at fixed concentration of 5'-FSBA, 0.5 mM; the activity was not increased proportionally. To determine the inhibition effect directly, the increment of activity in every one minute was calculated and plotted as a function of time (shown in figure 5-b). Activity at 0 minute was derived from the one minute activity with DMF, without 5'-FSBA at each different ATP concentration. Figure (5)-b showed that 5'-FSBA inhibited the enzyme in two different phases; up to one minute, inhibition was very fast (half time less than 1 minute) and later inhibition was occurring rather slowly (half time about 5 to 6 minutes). These two distinct inhibition rates could imply that the inhibiton might occur in two different ways.

Figure (5)-a. Time course with 5'-FSBA

      Figure (5)-a shows the time course with 5'-FSBA present at 0.5 mM and two different ATP concentrations of 0.75 mM and 1.5 mM. In a reaction vial which contains 0.5 mM of 5'-FSBA and reagents for enzyme assay including 50 ul of AV I, 50 ul of AV II was added and reacted with 5'-FSBA in two different ATP concontrations. After every minute interval of addition of AV II, reaction was stopped and activity was measured.

               O :1.5 mM ATP, 0.5 mM5'-FSBA

               ■ : 0.75 mM ATP, 0.5 mM 5'-FSBA
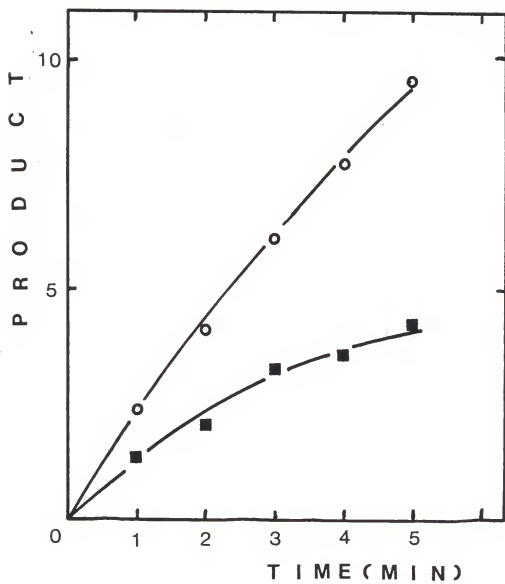
LEGEND to Figure (5)-b

Figure (5)-b. Time course of 5'-FSBA inhibition

Figure (5)-b shows the time course 5'-FSBA inhibition. From the result of Fig. (5)-a, the increment of activity in every one minute was calculated and plotted. The activity at 0 minute was derived from the activity with DMF at different ATP concentration.

O : 1.5 mM ATP, 0.5 mM 5'-FSBA

■ : 0.75 mM ATP, 0.5 mM 5'-FSBA
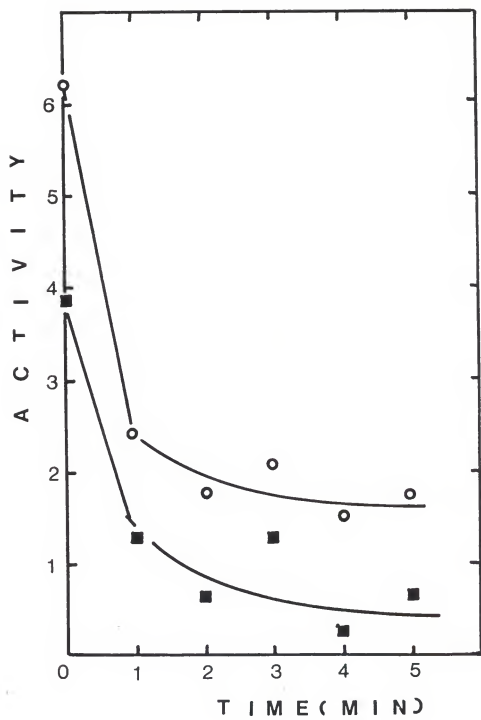
(6) Initial Fast Inhibition As A Function Of [ATP] Or [5'-FSBA]

a. Initial inhibition as a function of [ATP]

The results are summarized in table 7. To get a relative inhibition in different ATP concentrations (0.25, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0 mM), the ratio $\dfrac{\text{activity with 5'-FSBA}}{\text{activity with DMF control}} \times 100(\%)$ was calculated and plotted as a function of ATP concentration (see figure 6-a). 5'-FSBA inhibition was bigger at lower ATP concentration; at 0.25 mM ATP, about 80% activity was inhibited and at 1.0 mM ATP, about 55% activity was inhibited. As the concentration of ATP increased, the inhibition by 5'-FSBA was decreased; at more than 5 mM of [ATP], 5'-FSBA was likely to have no inhibition effect. From the result that high concentration of ATP protects AV II from the inhibition by 5'-FSBA, it is suggested that 5'-FSBA might compete with ATP in initial reaction. When this result was plotted in a Dixon plot, 1/v vs [I], it showed that 5'-FSBA was neither a pure competitive inhibitor nor a pure non-competitive inhibitor; it showed a competitive inhibititor pattern between low ATP concentrations (i.e., 1.0 mM and 1.5 mM of ATP) and it also showed a non-competitive pattern between high ATP concentrations (i.e., 1.5 mM and 2.5 mM of ATP). 5'-FSBA was likely to be a mixed inhibitor, reacting with more than one form of the enzyme.

Table 7. Initial % Inhibition As A Function Of [ATP]

[ATP] (mM)	C: DMFcontrol (nmole/2min)	T:1.0 mM 5'-FSBA (nmole/2min)	T/C x100 (%)
0.25	32.35	7.6	23.5
1.0	61.30	25.78	42.0
1.5	76.05	49.80	65.5
2.5	66.60	50.70	76.1
5.0	58.90	66.60	113.0
7.5	63.80	51.10	80.0
10.0	49.8	44.1	88.0

b. Initial Inhibition As A Function Of [5'-FSBA]

The results are shown in Dixon plots, 1/v vs [I] at two different fixed ATP concentrations; figure 6-b shows a plot of 0.25 mM and 0.5 mM ATP concentration and figure 6-c shows a plot of 0.5 mM and 1.0 mM of ATP concentration. Although the same enzyme was used for these two set of experiments, the experiments were not performed in same day. Since enzyme loses its activity during the process of repeated freezing and thawing, the results of two experiments could not compared in a same scale. Both figure 6-b and 6-c show a competitive inhibitor pattern and Ki, the binding constant ot 5'-FSBA on AV II was graphically obtained which was approximately 0.5 mM. This Ki value is almost the same as the reported Km value of MgATP to Fe protein which is 0.5 mM (1), indicating that 5'-FSBA competes with MgATP having a similar affinity at low ATP concentrations.

LEGEND to Figure 6-a

Figure 6-a. Initial % inhibition as a function of ATP

      Figure 6-a shows the initial % inhibition as a function of
[ATP]. At several different ATP concentrations (0.25, 1.0, 1.5,
2.5, 5.0, 7.5, 10.0) with fixed 5'-FSBA concentration of 1.0 mM or
with DMF, 50 ul of AV II was added and 2 minutes after reaction was
stopped. % inhibition was calculated as follows and plotted as a
fuction of [ATP].

$$\frac{T \text{ (activity with 5'-FSBA)}}{C \text{ (activity with DMF)}} \times 100, \text{ at each ATP concontration}$$
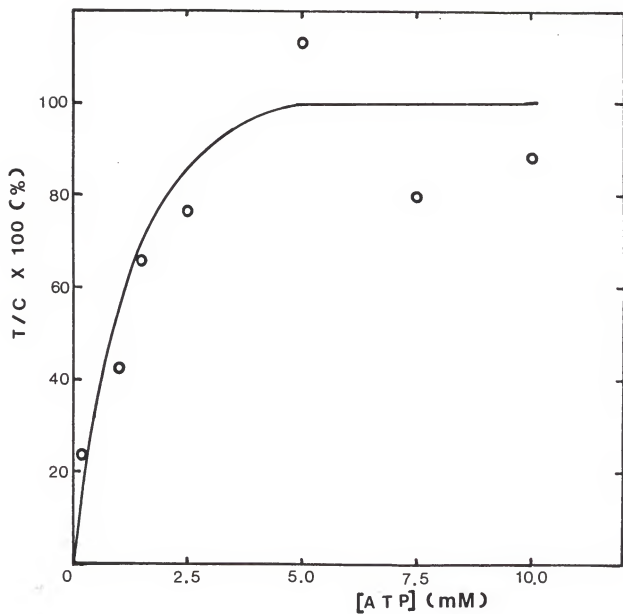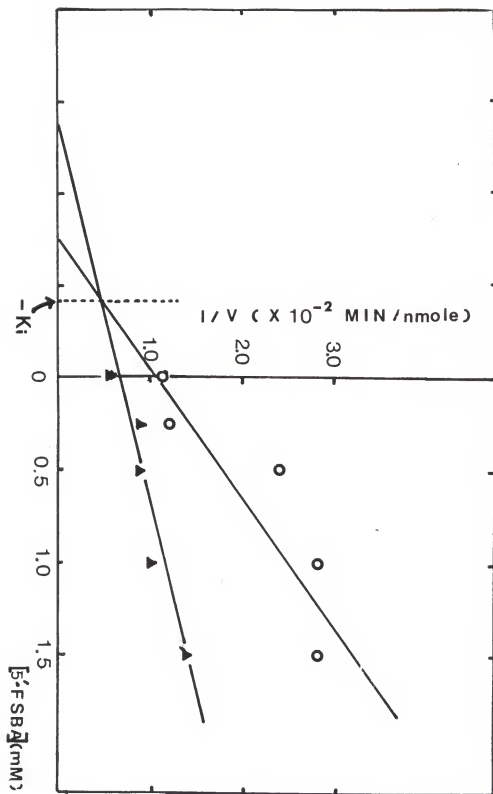
Figure 6-b. Dixon plot of initial inhibition (I)

      Figure 6-b shows the Dixon plot at two different fixed concentrations of ATP, 0.25 and 0.5 mM. In reaction vials of several different 5′-FSBA concentrations, 50 ul enzyme mixture was added and activity was measured after 2 minutes. The Ki value was obtained graphically, approximately 0.5 mM.

            0.25 mM ATP   --O--

            0.5 mM ATP   --▲--

LEGEND to Figure (6)-c

Figure 6-c. Dixon plot of initial inhibition (II)

Figure 6-c shows the Dixon plot of two different fixed ATP concentrations, 0.5 and 1.0 mM. The Ki value was obtained, approximately 0.5 mM.

0.5 mM ATP	--- ▲ ---
1.0 mM ATP	--- ☐ ---
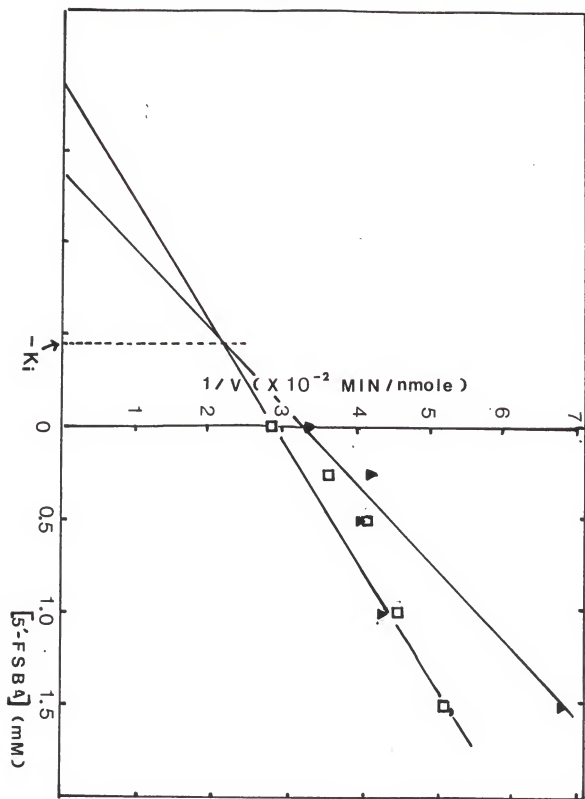
(7) Rate Of Irreversible Inhibition

The results are summarized in Table 8. The remaining activity after increasing of [ATP] was calculated per unit reaction time and this data was plotted as a function of time. 0 time activity was the DMF control activity for 1 minute at 2.5 mM ATP concentration. Figure 7 shows the rate of irreversible inhibition; half time was about 7-8 minutes. This irreversible inhibition was relatively slow compared to the initial fast inhibition whose half time was less than 1 minute (figure 5-b). This difference could suggest that initial fast inhibition might occur in a different way, i.e., reversible inhibition competing with ATP. This irreversible inhibition rate was comparable to the rate of initial inhibition with MgADP alone whose half time was about 7 minutes (figure 4).

These overall data suggest that 5'-FSBA inhibition on Fe protein of *Azotobacter vinelandii* was occurring in two different steps; fast reversible inhibition competing with ATP followed by relatively slow irreversible inhibition on AV II.

Table 8. Determination Of Rate Of Irreversible Inhibition

time	products formed with 0.5 mM ATP (nmole)	products formed with 0.5 mM + 2.5 mM ATP (nmole)	net activity in second stage (nmole/min)
1 min	6.4	77.6	77.6-6.4/5 = 14.24
2 min	11.75	61.6	61.6-11.75/5 = 9.97
3 min	14.5	68.3	68.3-14.5/5 = 10.76
4 min	18.6	61.65	61.65-18.6/5 = 8.61
5 min	19.5	60.77	60.77-19.5/5 = 8.25

LEGEND to figure (7)

Figure 7. Rate of irreversible inhibition

Fig.7 shows the rate of irreversible inhibition on AV II by
1.0 mM of 5'-FSBA (see text for detail).
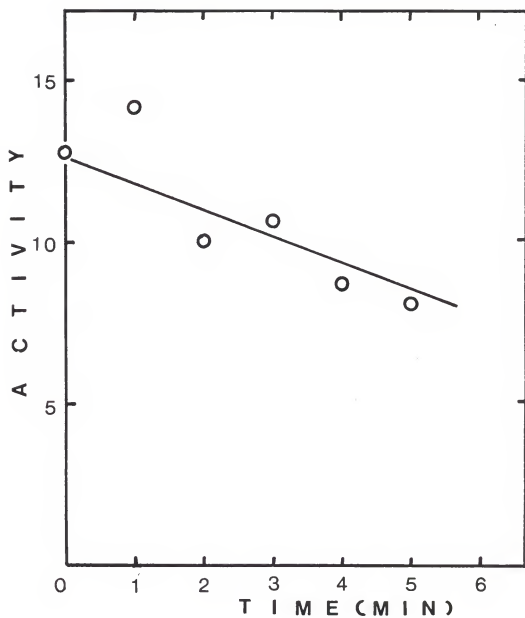
(8) Fe detection of AV II in the presence of 5'-FSBA by α,α' - dipyridyl

The results are shown at figure 8 and summarized in table 9.The absorbance at (a) was measured for blank and increased absorbanse by adding α,α'-dipyridy at (b) was due to the released Fe from preexisting denatured protein. The measured absorbance by adding DMF or 5'-FSBA was regared as 0 min absorbance. The result showed that in 10 minutes after adding 5'-FSBA, a large absorbance change occurred (0.426 - 0.372 = 0.054); this absorbance change was approximately equal to 0.0064 mM of Fe since molar absorptivity of the colored product is 8,400. While, DMF control did not release Fe in 10 minutes, as expected (30). Since the protein used showed the activity of 1,000 nmole/min/ml and specific activity of component II is to be 2,000 nmole/min/mg, about 0.5 mg/ml of protein was active in the enzyme used; this is approximately 0.008 mM (molecular weight of component II is 60,000). Therefore the absorbance change in 10 minutes was caused by about 0.8 Fe released (0.0064 mmole Fe/0.008 mmole protein = 0.8 Fe/protein).

After 30 minutes, DMF control released Fe almost same rate as 5'-FSBA treated. Since reduced AV II with MgADP is not supposed to release Fe (30), this result is not expected. Absolute absorbance change of 5'-FSBA treated could not be calculated as released Fe since control was not constant after 30 minutes. Probably uncontrolled temperature could be one reason to increase the absorbance of control; the use of spectrophotometer for a long

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time could heat the detector and increase the baseline of
absorbance. Subtracting the increased absorbance of control from
that of 5'-FSBA treated, 5'-FSBA caused no further of Fe released
after 10 min. This result showed that 5'-FSBA might affect the
comformation around the iron sulfur center and expose the iron
sulfur center to chelator in the beginning of its reaction.

Table 9. Fe Detection Of AV II With 5'-FSBA

```
-----------------------------------------------------------------
                                   Absorbance at 520 nm
      Time                  C (DMF control)   T (5'-FSBA treated)
-----------------------------------------------------------------

a.  MgADP, buffer,              0.201             0.276
    AV II, DTH added

b.  α,α' -dipyridyl added       0.330             0.360

c.  DMF or 5'-FSBA added        0.348             0.372
    (0 min)
d.  5   min                     0.348             0.426

e.  10 min                      0.350             0.436

f.  20 min                      0.354             0.444

g.  30 min                      0.358             0.454

h.  60 min                      0.382             0.480

i.  90 min                      0.408             0.502

-----------------------------------------------------------------
```

This result shows that chelation by α,α' -dipyridyl could provide
the way to determine whether 5'-FSBA inhibition was caused by the
change of iron sulfur center activity or not. But to get a more

reliable data, this experiment should be done more carefully,
considering the followings.

   a) Temperature should be controlled at 25°C constantly.

   b) Purification of enzyme may be helpful to get an accurate
      measurement.

   c) It would be necessary to confirm that the measured
      absorbance reflected the total Fe released for precise
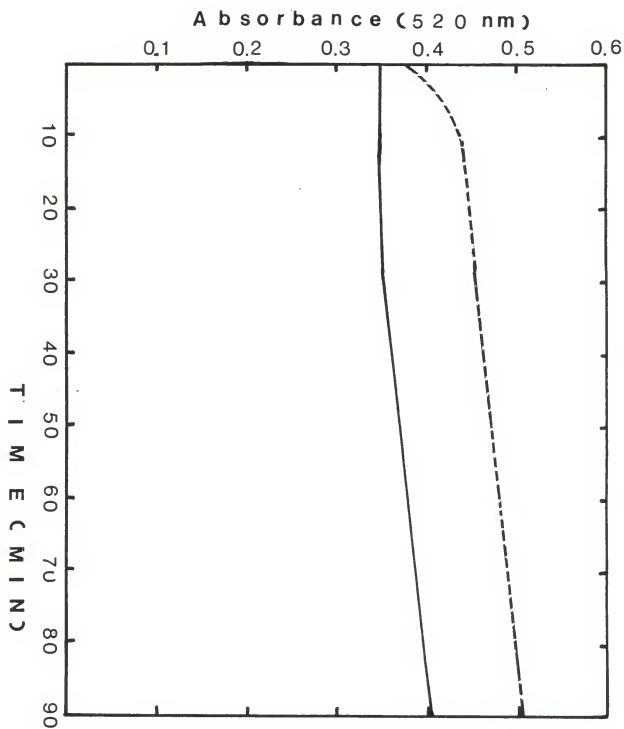   quantitative interpretation of data.

Figure 8. Fe chelation by α,α' -dipyridyl

Figure 8 shows the absorbance at 520 nm with reduced AV II, and α,α'-dipyridyl in pH 8.0, 50 mM Tris.HCl buffer containing 0.1 M Nacl in two different conditions; with 5'-FSBA or DMF.

     —— : reduced AV II + MgADP + DMF

     --- : reduced AV II + MgADP + 5'-FSBA

CONCLUSION

The Iron protein of *Azotobacter* *vinelandii* nitrogenase was studied using an ATP analog, 5'-Fluorosulfonyl benzoyl adenosine (5'-FSBA). 5'-FSBA inhibited the Fe protein but not the MoFe protein. Since 5'-FSBA contains electrophilic sulfonyl fluoride group, the specificity of 5'-FSBA inhibition on AV II was tested; phenyl methane sulfonyl fluoride (PMSF) which contains the same sulfonyl fluoride group does not inhibit the AV II, indicating that 5'-FSBA has a specific inhibition effect on AV II due to its structural analogy to ATP.

Dr Davis's earlier observation that 5'-FSBA inhibition was stronger in the presence of MgADP than 5'-FSBA alone was consistent with my following result. At high ATP concentration, 5'-FSBA did not inhibit the enzyme. However, when the reaction continues for a longer time, creatine phosphate is running out and the equilibrium of ATP regenerating reaction is no longer overwhelmingly in the favor of ATP production, resulting the increase of the concentration of MgADP. When the MgADP concentration is increased for a longer reaction time, 5'-FSBA begins to inhibit the AV II (data are not shown).

5'-FSBA inhibition on Fe protein was occurring in two different steps. It could be treated as instantaneous reversible inhibition competing with ATP followed by relatively slow (half time about 7 minutes) irreversible inhibition on AV II. The

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result of Dixon plot showed that 5'-FSBA is a mixed inhibitor reacting with more than one form of enzyme. Ki value was obtained form the Dixon plot between low ATP concentrations. The Ki obtained was approximately the same as the reported Km of AV II for MgATP indicating that 5'-FSBA competes with MgATP having a similar affinity.

Irreversible inhibition on AV II by 5'-FSBA could occur in several possible ways; modification of active amino acid residue or comformational change of iron sulfur center could be one of them. The latter possibility was tested using the Fe chelator, α,α'-dipyridyl. 5'-FSBA caused about one mole of Fe release out of four per protein molecule in 10 minutes, whereas DMF control did not release Fe, indicating 5'-FSBA inhibition may relate to the changing activity of iron sulfur center. However the rate of Fe release was not constant; very quick Fe release in 10 minutes (about 0.8 mole of Fe per mole or protein), slow Fe release in 30 minutes (0.2 mole Fe release between 10 minutes and 30 minutes), and no net Fe release after 30 minutes. Therefore more thorough experiment should be performed to conclude the relationship between the irreversible inhibition and the change of iron sulfur center activity and a more precise quantitative interpretation should be supported.

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THE INTERACTION OF 5'-FLUOROSULFONYL BENZOYL ADENOSINE

WITH IRON PROTEIN OF *AZOTOBACTER VINELANDII* NITROGENASE

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The Iron protein of *Azotobacter vinelandii* nitrogenase was studied by an ATP analog, 5'-Fluorosulfonyl benzoyl adenosine (5'-FSBA). 5'-FSBA was a specific inhibitor to the Fe protein but not to theMoFe protein. The inhibition by 5'-FSBA was occurring in two different ways; instantaneous reversible inhibition competing with ATP followed by irreversible inhibition. 5'-FSBA was likely to be a mixed inhibitor, reacting more than one form of the enzyme. The Ki value was obtained between low ATP concentrations; it showed that 5'-FSBA competes with MgATP having a similar affinity.

The relationship between irreversible inhibition and the change of iron sulfur center activity was examined using an iron-chelating agent, α, α'-dipyridyl.